AD_____

Award Number: DAMD17-02-1-0651

TITLE: Signaling Pathways Controlling the Growth and Proliferation of Drosophila Perineurial Glial Cells

PRINCIPAL INVESTIGATOR: Michael Stern, Ph.D.

CONTRACTING ORGANIZATION: Rice University
Houston, Texas 77251-1892

REPORT DATE: May 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2004	Annual (1 May 03 - 30 Apr 04)

4. TITLE AND SUBTITLE
Signaling Pathways Controlling the Growth and Proliferation of Drosophila Perineurial Glial Cells

5. FUNDING NUMBERS
DAMD17-02-1-0651

6. AUTHOR(S)

Michael Stern, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Rice University
Houston, Texas 77251-1892

E-Mail: stern@rice.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The long term goals of this research are to understand the mechanisms by which *NF1* controls growth using the Drosophila peripheral nerve. This system is advantageous because we can apply a number of powerful molecular genetic methodologies that are not available in other systems. The aims of this project address three specific aspects of growth control. In our first aim, we asked if *NF1* acts downstream of a G protein to exert its effects. We have found that overexpression of *NF1* and its upstream activators *amnesiac (amn)* and $G_{\alpha}s$, each confer a similar glial growth phenotype: enhancement of the effects on glial growth of expression of Ras^{V12}, but no effect in an otherwise wildtype background. These data strongly support the hypothesis that Neurofibromin is an effector of a signalling pathway acting by *amn* through $G_{\alpha}s$, Last year, we reported negative results for our second aim, but these negative results can now be reinterpreted based on the success of our newly revised hypothesis. Under the aegis of this hypothesis, new and more productive experiments to test the effects of altered neurotransmitter release on perineurial glial growth are proposed. The third aim was completed last year and no new results are presented.

14. SUBJECT TERMS
molecular genetics, neuroscience, cell biology, cell signaling

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Over the last several years, my lab has been developing the Drosophila peripheral nerve as a system with which to identify and study the signalling pathways controlling growth of the perineurial (outer) glial layer (Yager et al., 2001). The idea behind this approach is to apply the various molecular genetic methodologies uniquely available in Drosophila to enable us ultimately to identify all of the relevant genes that interact with *NF1* to control growth, and place *NF1* and these partner genes in as complete a mechanistic context as possible. Then this mechanism could be tested and refined in systems more similar to humans but more difficult to work with (i.e. the mouse). Because all of the experimentation is performed on the acutely dissected third instar larva, there are no complications or caveats associated with experimentation on cell culture systems, and we assay the entire nerve cross section as it exists within the whole organism. We thought that a more complete mechanistic understanding of growth control within peripheral nerves would greatly facilitate the ability to design drugs able to combat neurofibromas. Within this larger context, the specific research being performed under this grant was designed to test particular hypotheses that would increase this mechanistic understanding. The first task was designed to test the hypothesis that the *amnesiac*-encoded neuropeptide acts upstream, and Neurofibromin acts downstream, of a G protein subunit. The second task proposed additional experiments to test the hypothesis that perineurial glial growth is regulated by neurotransmitter release from motor neurons. The third aim was designed to test the possibility that growth and mitosis could be mechanistically uncoupled. Successful completion of these aims would provide important information concerning the control of growth within peripheral nerves at the molecular level.

BODY

Task one: Does Neurofibromin act downstream of a G protein to control perineurial glial growth? In this task I proposed to test the hypothesis that perineurial glial growth is negatively regulated by the *amnesiac (amn)*-encoded neuropeptide acting through the G protein $G_{\alpha}s$, Neurofibromin and Pushover. As I reported last year, initial experiments to test this hypothesis gave negative results. However, at the same time, data on a related project funded by my NIH grant suggested that this hypothesis might be incomplete. In particular, we found that expression of a constitutively active protein kinase A (PKA*) enhanced the increased perineurial glial growth conferred by expression of the constitutively active Ras^{V12}. Furthermore, we found that the $NF1^{P2}$ mutation actually suppressed the increased perineurial glial growth conferred by Ras^{V12} (Yager et al., in preparation, supplied in appendix). These results are not possible to explain with the hypothesis as originally proposed and thus the hypothesis was revised. Taken together, the data suggest that *NF1* (and hence, by extension, Amn and $G_{\alpha}s$) has dual, opposing, roles in controlling perineurial glial growth: *amn, NF1* and $G_{\alpha}s$ activity increase perineurial glial growth via activation of PKA, whereas *amn, NF1* and $G_{\alpha}s$ activity reduce perineurial glial growth via inhibition of Ras.

This revised hypothesis suggests that overexpression of either *amn*, a constitutively active $G_{\alpha}s$ called $G_{\alpha}s$*or *NF1* should each hyperaactivate PKA and thus enhance the effects of Ras^{V12} on growth, Furthermore, this hypothesis suggests that the $NF1^{P2}$ mutation should be epistatic to overexpressed *amn* and $G_{\alpha}s$*, but that overexpressed *PKA** should epistatic to $NF1^{P2}$. During the past year we have been testing several aspects of this revised hypothesis, and so far, every piece of data we have collected strongly support this revised hypothesis. Below I show the effects on perineurial glial growth of overexpression of the signalling molecules listed above in a Ras^{V12} background. Note that for ease of comparison, some data points are presented in multiple figures.

Effects of overexpression of *amn*: As can be seen in Figures 1 and 2, overexpression of *amn* significantly enhances the growth phenotype exerted by either of two *UAS-*Ras^{V12} transgenes: the strongly expressed *UAS-*Ras^{V12} located on chromosome III (Figure 1, compare lane 6 with lanes 5 and

7), and the weaker transgene (written as "*UAS-Ras*$^{V12(weak)}$, in Figure 2, compare *gli-amn; Ras*$^{V12(weak)}$, which is the third lane, with

Figure 1: Enhancement of the glial growth phenotype conferred by *Ras*^{V12} by overexpression of *amnesiac*

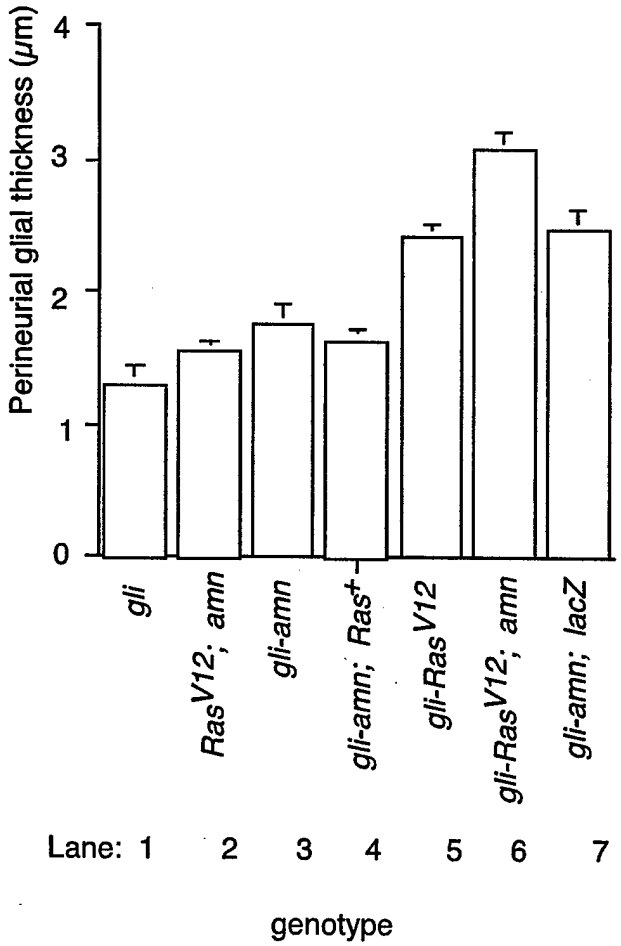

Figure 1: Overexpression of *amn* enhances the growth phenotypes conferred by *Ras*^{V12}. The genotypes are listed on the X axis according to the new nomenclature for *GAL4* and *UAS*-containing transgenes. In this nomenclature, the *GAL4* and *UAS-* prefixes are eliminated for clarity. For example, *gli-amn* (as written in lane 3) represents the data from larvae expressing both *gli-GAL4* and *UAS-amn*. Means and SEMs of perineurial glial thickness from the indicated genotypes are shown. The following combinations have statistically significant differences (two tailed, unpaired t-test). For lane 5 (n=72) versus lane 6 (n=22), p=0.0026. For lane 6 (n=22) versus lane 7 (n=23), p=0.008.

Figure 2: Enhancement of the glial growth phenotype conferred by a weakly expressed Ras^{V12} transgene by overexpression of *amnesiac*

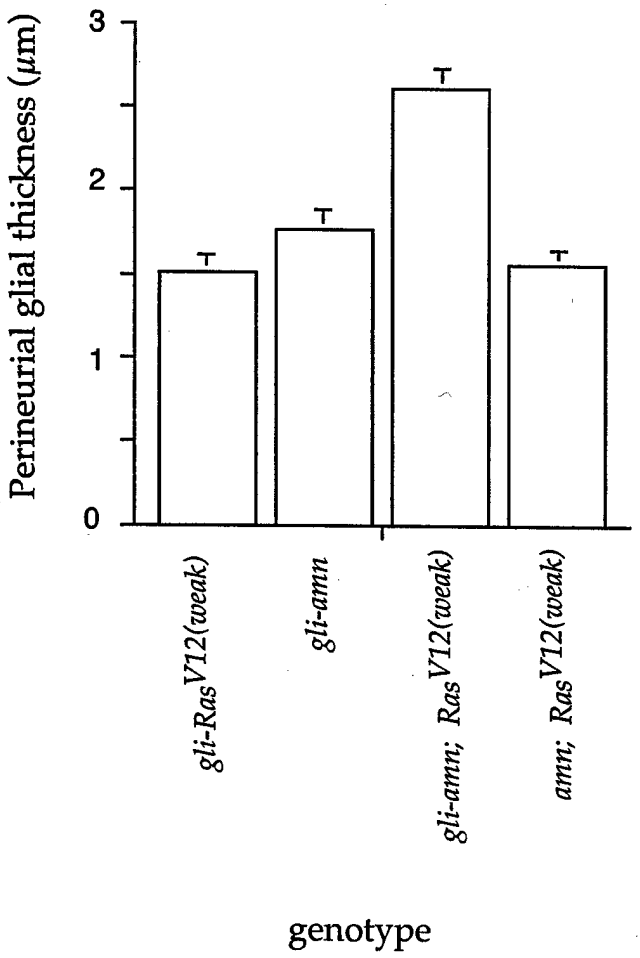

Figure 2: Overexpression of *amn* enhances the growth phenotypes conferred by a weak *UAS-*Ras^{V12} transgene (labelled *UAS-*$Ras^{V12(weak)}$). The genotypes are listed on the X axis. Means and SEMs of perineurial glial thickness from the indicated genotypes are shown. The following combinations have statistically significant differences (two tailed, unpaired t-test). For *gli-*$Ras^{V12(weak)}$ (n=29) versus *gli-amn;* $Ras^{V12(wea)k}$ (n=25), $p<0.0001$. For *gli-amn* (n=19) versus *gli-amn;* $Ras^{V12(weak)}$ (n=25), $p<0.0001$. For *amn;* $Ras^{V12(weak)}$ (n=29) versus *gli-amn;* $Ras^{V12(weak)}$ (n=25), $p<0.0001$.

perineurial glial thickness values in the other lanes). In contrast, overexpression of *amn* does not confer increased perineurial glial growth to larvae expressing *UAS-*Ras^{+}(in Figure 1, compare lane 4 with lane 6, and with lanes 1-3), or increase perineurial glial thickness in the absence of any *UAS-Ras* transgene. These phenotypes are very similar to what is conferred by expression of a constitutively active PKA. Thus, we interpret the enhancement of Ras^{V12} by *amn* overexpression to result from an *amn*-induced increase in PKA activity, which also enhances the effects of Ras^{V12}. These data strongly support the hypothesis that *amn* activates perineurial glial growth by activating PKA activity in the peripheral glia, possibly in an *NF1*-dependent manner.

In contrast to the enhancement of growth conferred by overexpression of *amn*, when we co-expressed an "indifferent" enzyme (β-galactosidase, encoded by *UAS-lacZ*) with Ras^{V12}, we observed no enhancement (Figure 1, compare lane 7 with lane 5). This control experiment demonstrates that the the Ras^{V12} phenotype is not enhanced merely by the presence of a *UAS*-driven transgene, but apparently requires the increased PKA activity conferred by *amn*-overexpression.

Our studies on the effects of *amn* overexpression are almost complete. The only experiment remaining is to test the prediction that *amn* activates PKA via *NF1*. This prediction suggests that *NF1* will be epistatic to *amn*, and thus that in the presence of $NF1^{P2}$, the effects of Ras^{V12} on perineurial glial growth will be suppressed even when *amn* is overexpressed. This prediction will be tested in the final year of funding.

Effects of overexpression of $G_{\alpha}s^*$: As reported last year, larvae expressing $G_{\alpha}s^*$ in peripheral glia have poor viability, and it is difficult to obtain third instar larvae of this genotype. However it is not impossible to obtain such larvae, With effort, we have succeeded in beginning to study the effects of overexpression of $G_{\alpha}s^*$ in combination with expression of Ras^{V12}. as well as in an otherwise wildtype background. As shown in figure 3 below, we found that expression of $G_{\alpha}s^*$ in an otherwise wildtype background has no significant effect on perinurial glial thickness (second lane), but significantly enhances the effects of Ras^{V12} (compare the third and fourth lanes) which is a phenotype identical to that conferred by overexpression of *amn* or *PKA**.

Figure 3: Enhancement of the glial growth phenotype conferred by Ras^{V12} by overexpression of $G_{\alpha}s^*$

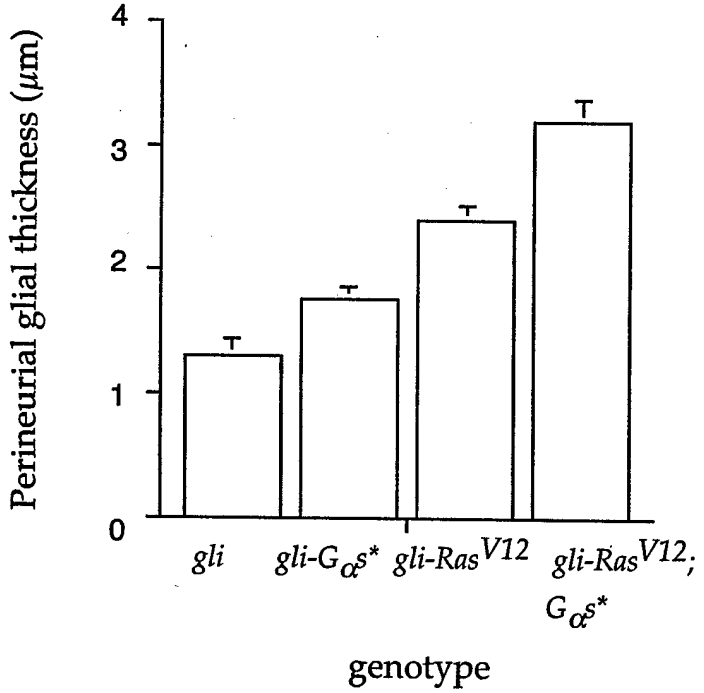

Figure 3: Overexpression of $G_{\alpha}s^*$ enhances the growth phenotypes conferred by Ras^{V12}. Means and SEMs of perineurial glial thickness from the indicated genotypes are shown. The following combinations have statistically significant differences (two tailed, unpaired t-test). For *gli-*Ras^{V12}*;* $G_{\alpha}s^*$ (n=25) versus *gli-*Ras^{V12} (n=72), p=0.0004, versus *gli-* $G_{\alpha}s^*$ (n=29), p<0.0001, versus *gli* (n=13), p<0.0001.

Our studies on the effects of overexpression of $G_{\alpha}s$* are incomplete. We still need to test the effects of $G_{\alpha}s$*overexpression in a background of overexpressed Ras^{+} and the weakly expressed $Ras^{V12(weak)}$ as described above for *amn* overexpression. We also need to test the prediction that the *NF1* null mutation $NF1^{P2}$ is epistatic to overexpression of $G_{\alpha}s$*. These experiments will be performed in the final year of this award.

Effects of overexpression of *NF1*: As shown in Figure 4, overexpression of *NF1* within peripheral glia in an otherwise wildtype background has no effect on perineurial glial growth. This lack of effect was conferred by overexpression induced by two independent *UAS-NF1* transgenes (both supplied by Andre Bernards; Figure 4 compare lanes 2 and 3 with lane 1). In contrast, overexpression of *NF1* significantly enhanced the perineurial glial growth phenotype conferred by expression of Ras^{V12} (Figure 4, compare lanes 4 and 5), which is precisely the result observed when *amn*, $G_{\alpha}s$* and *PKA**, the other transgenes predicted to increase PKA activity, and is precisely the result predicted by the newly revised hypothesis described above.

Our studies on the effects of overexpression of *NF1* are incomplete. We still need to test the effects of *NF1* overexpression in a background of overexpressed Ras^{+} and the weakly expressed $Ras^{V12(weak)}$ as described above for *amn* overexpression.

Figure 4: Enhancement of the glial growth phenotype conferred by Ras^{V12} by overexpression of *NF1*

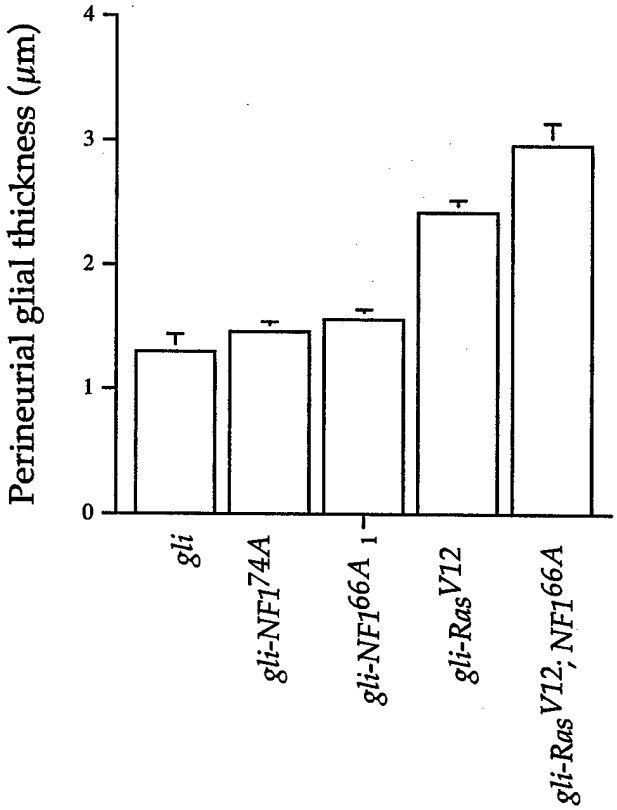

Figure 4: : Overexpression of *NF1* enhances the growth phenotypes conferred by Ras^{V12}. Means and SEMs of perineurial glial thickness from the indicated genotypes are shown. The following combinations have statistically significant differences (two tailed, unpaired t-test). For *gli-*Ras^{V12}*; NF1* (n=32) versus *gli-*Ras^{V12} (n=72), p=0.01.

In conclusion, all of our experiments performed during the past year have supported the newly revised hypothesis, giving me more confidence that this hypothesis is likely to be correct. In the final year of funding, I propose to complete these studies. Most importantly, I propose to test the prediction that the thickened phenotype conferred by *UAS-*Ras^{V12} and *UAS-PKA** is epistatic to $NF1^{P2}$; that is, that the increased thickness of Ras^{V12} and PKA* will be observed both in an $NF1^{+}$ and $NF1^{P2}$ background. We will also test the prediction that the $NF1^{P2}$ null mutation will be epistatic to both *UAS-amn* and *UAS-*$G_{\alpha}s$*. If these predictions are confirmed by experiment, then the hypothesis that a signalling pathway in which Amn activates PKA via $G_{\alpha}s$ and Neurofibromin, will be confirmed.

Task two: Further tests of the hypothesis that increased neurotransmitter release from motor neurons (or increased neurotransmitter persistence) affects perineurial glial growth. During the first year of funding, we began addressing this issue. As reported last year, we measured perineurial glial thickness in two triple mutants: *eag Sh;* $NF1^{P2}$, and *eag; ine;* $NF1^{P2}$. In contrast to the prediction, we found that there was no significant increase in perineurial glial thickness in the triple mutants compared to the double mutants. Because of these negative findings, we put this task to the side during the previous funding year in order to concentrate on task one, on which we were able to make excellent progress as described above.

Based on the successes of the newly revised hypothesis, I can now re-interpret the negative findings from task two. The newly revised hypothesis suggests that *NF1* has dual, opposing roles on perineurial glial growth. These opposing roles obscure the ability of genetic alterations to induce phenotypic effects and complicates efforts to infer mechanism. In this view, we can best observe phenotypic effects of increased neurotransmitter release by combining the *eag Sh* and *eag; ine* mutations with transgenes that have only single roles on perineurial glial growth. The best transgenes for this purpose would be the *Ras*^{V12}and *PKA** transgenes, which as far as we can tell, each activate perineurial glial growth without any growth-inhibitory activities. Therefore, I propose to continue this task in a slightly restructured form. I propose to compare perineurial glial thickness in larvae expressing *Ras*^{V12} or *PKA**, and also carrying the *eag, ine, Sh, eag Sh,* and *eag; ine* mutations or double mutations. I think that in either the *Ras*^{V12} or *PKA** backgrounds, we will observe increased glial growth conferred by the *eag; ine* and *eag Sh* double mutants.

Task three: Can perineurial glial growth be genetically uncoupled from perineurial glial proliferation? This task was completed during the first year of funding. Confusing issues regarding the ability of *Ras*^{V12}, but not *amn* or *ine;* $NF1^{P2}$ double mutants to increase perineurial glial nuclei number, are now explained by the observation that *amn* and *NF1* each affect signalling pathways (i.e. PKA) in addition to Ras.

KEY RESEARCH ACCOMPLISHMENTS

We demonstrated that the *amnesiac*-encoded neuropeptide, $G_{\alpha}s$, and Neurofibromin enhance the effects of *Ras*^{V12} on perineurial glial growth. This discovery supports the hypothesis in task one that a G protein acts upstream of Neurofibromin in the control of perineurial glial growth.

We generated evidence that Amn, $G_{\alpha}s$, and Neurofibromin activate PKA in Drosophila peripheral glia.

REPORTABLE OUTCOMES

1. Presentation entitled " Ras activity in peripheral glia promotes perineurial glial growth in Drosophila peripheral nerves", by James C. Yager, Alexander Rottgers, Michelle C. Wells, Elizabeth L. Carter and Michael Stern, was presented in a platform session at the NNFF International Consortium meeting, held at Aspen, CO, in June, 2003.

2. Abstract entitled "Evidence that PI3 kinase mediates the effects of Ras on perineurial glial growth in Drosophila peripheral nerves" was accepted for oral presentation at the NNFF International Consortium meeting, to be held at Aspen, CO, May 23-May 25, 2004. Although not in evidence from the abstract title, during the first half of the talk I will present the data on interactions between Ras^{V12} and overexpression of *amn* and $G_{\alpha}s^{*}$ as described in the "Body" section above, under "Task one".

CONCLUSIONS

I report two major conclusions. First, using overexpression studies, we report that Neurofibromin activates perineurial glial growth (in larvae expressing Ras^{V12}) by activating PKA. This conclusion confirms the earlier observation that loss of function mutations in *NF1* suppress the increased glial growth conferred by Ras^{V12} expression and demonstrate that Neurofibromin has two, opposing roles in the regulation of perineurial glial growth. Second, again using overexpression studies, we report evidence that Amnesiac and $G_{\alpha}s$ act as upstream activators of Neurofibromin. The hypothesis that a neuropeptide and $G_{\alpha}s$ act upstream of Neurofibromin is not a new one, but it is a hypothesis that required testing. In fact, one important task of this idea award was precisely to test this hypothesis. Although this demonstration is not yet complete (most importantly epistasis testing is required for final confirmation), our results obtained during the past year (see task one) and anticipated results during the final year might finally demonstrate that this hypothesis is true. An understanding of the signals regulating the activity of Neurofibromin will not only add to our general knowledge of nerve growth control, but also improve our ability to select useful pharmacological agents for treatment.

REFERENCES

Yager, J., Richards, S., Hekmat-Scafe, D.S., Hurd, D.D., Sundaresan, V., Caprette, D.R., Saxton, W.M., Carlson, J.R., and Stern, M. (2001). Control of *Drosophila* perineurial glial growth by interacting neurotransmitter-mediated signaling pathways. Proc. Natl. Acad. Sci. U.S.A. *98*, 10445-10450.

Yager, J., Rottgers, A., Caldwell, P., Wells, M, Lavery, W. and Stern, M. Ras and PKA activity in peripheral glia promote perineurial glial growth in Drosophila peripheral nerves, in preparation.

APPENDIX

1) Abstract of presentation to the NNFF Consortium on NF1 and NF2 (Aspen, CO, May, 2004).

CONTACT INFORMATION

Michael Stern
Dept. of Biochemistry MS-140
Rice University
PO Box 1892

Houston, TX 77251-1892
stern@rice.edu
(713) 348-5351
FAX: (713) 348-5154

ABSTRACT FORM

TOPIC: Signaling pathways in NF and TSC

TITLE: Evidence that PI3 Kinase mediates the effects of Ras on perineurial glial growth in Drosophila peripheral nerves

William Lavery, Michelle C. Wells and Michael Stern

Position of presenting author: PI

Affiliation: Dept. of Biochemistry and Cell Biology, Rice University
Address: Dept. of Biochemistry MS-140, Rice University, PO Box 1892, Houston, TX 77251.
Tel: (713) 348-5351 Fax: (713) 348-5154 Email: stern@bioc.rice.edu

Drosophila peripheral nerves comprise a layer of motor and sensory axons, wrapped by an inner peripheral glia (analogous to the mammalian Schwann cell) and an outer perineurial glia (analogous to the mammalian perineurium). We have been using these nerves as an assay platform to test the effects of mutations and transgenes on perineurial glial growth. It was previously shown that perineurial glial growth in third instar larval nerves is regulated by a number of genes including *push*, which encodes a large Zn^{2+}-finger-containing protein, *amn*, which encodes a putative neuropeptide related to PACAP, and *NF1*. We found that expression of the constitutively active Ras^{V12} transgene specifically in peripheral glia increased growth within the perineurial glia. This result demonstrates that Ras activity is sufficient to promote perineurial glial growth, and that Ras can act cell nonautonomously. Surprisingly, we found that the $NF1^{P2}$ null mutation suppresses these effects of Ras^{V12}, suggesting that *NF1* has a relevant activity that promotes, rather than inhibits, perineurial glial growth. The possibility that activation of adenylate cyclase represents this second activity is supported by the observation that expression within peripheral glia of any of three genes expected to increase protein kinase A (PKA) activity (a constitutively active PKA, the *amn*-encoded PACAP-like neuropeptide, or a constitutively active $G_{\alpha}s$) strongly enhances the growth promoting effects elicited by Ras^{V12} alone. These results are consistent with the possibility that a signalling pathway from the Amn neuropeptide through $G_{\alpha}s$, Neurofibromin, and PKA strongly potentiates the effectiveness of constitutive Ras activity on perineurial glial growth.

To identify the downstream components that mediate the effects of Ras, we tested the effects of constitutively active *Raf* and *PI3 Kinase* transgenes on perineurial glial growth. We found that expression of a constitutively active *PI3 Kinase,* but not a constitutively active *Raf*, strongly increased perineurial glial growth, suggesting the possibility that PI3 Kinase is an important mediator of the growth-promoting effects of Ras in peripheral nerves.